

5794

Influence of Preparative Method on the Lipid Profiles of Bovine Fat Globule Membrane

ABSTRACT

The lipid profiles of bovine fat globule membrane preparations were obtained using a procedure that separates neutral and polar lipids on a silica cartridge, analyzes components of these two lipid groups by high performance liquid chromatography, and identifies fatty acids of total lipid as well as neutral and polar lipids separately by gas chromatography. Fat globule membrane preparation involved either churning the cream, followed by ammonium sulfate precipitation of membrane, or a combined isolation-extraction with dimethyl sulfoxide. In preparations that did not include a delipidation step, the carryover of neutral lipids was significant. In contrast, preparations that were delipidated after ammonium sulfate precipitation or as part of the isolation-extraction had much lower levels of neutral lipids. For the latter samples, values for neutral and polar lipids were comparable to those reported previously. The relative ease with which fat globule membrane is obtained using the isolation-extraction procedure makes it the preferred method for subsequent isolation and purification of fat globule membrane proteins.

INTRODUCTION

The fat globule membrane (FGM) surrounding milk fat droplets during secretion from the lactating mammary cell is composed of plasma

*To whom correspondence should be addressed.

*Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

membrane from the apical portion of the cell and a third layer or coat of unknown origin (Patton & Keenan, 1975; Freudenstein *et al.*, 1979). Typical total membrane lipids (Thompson *et al.*, 1961; Keenan *et al.*, 1970; Keenan & Huang, 1972; Patton & Keenan, 1975; Sharma *et al.*, 1987) and a variety of proteins (Patton & Keenan, 1975; Dapper *et al.*, 1987) are present in FGM. Basch *et al.* (1976; 1985) identified the molecular weights of 15 individual FGM proteins, of which several were always present regardless of preparative method. Specific proteins identified in FGM include alkaline phosphatase (Morton, 1953), xanthine oxidase (Mangino & Brunner, 1977), butyrophilin (Heid *et al.*, 1983), and glycoprotein B (Basch *et al.*, 1976). Of special interest is the recent discovery that several FGM proteins possess a glycopospholipid anchor (Malin & Basch, 1990), as do many proteins of other animal membranes.

Although the lipids of FGM have been well characterized, the ratio of total lipids to protein varies with the method of FGM preparation (Kanno & Kim, 1990). For example, during our attempts to isolate and purify alkaline phosphatase from FGM, for studies of reversible thermal denaturation, we discovered that neutral lipids associated with FGM prepared by traditional methods interfered with protein purification procedures based on ion exchange and affinity chromatography (unpublished data). In contrast, FGM prepared by a more recent method (Dapper *et al.*, 1987) was free of these difficulties. Because the proteins of FGM are under continued investigation, it was appropriate to characterize in detail the lipid profile of FGM prepared by the newer procedure and compare it with that of FGM prepared by more traditional methods.

EXPERIMENTAL

Materials

Neutral lipid standards (NuChek Prep, Elysian, MN) and polar lipid standards (Avanti Polar Lipids, Birmingham, AL, or Sigma Chemical Co., St. Louis, MO) were stored in sealed vials at -20°C . Reference fatty acid methyl esters (FAMES) M-100 were purchased from NuChek Prep. HPLC-grade solvents were obtained from Burdick and Jackson (Muskegon, MI) and silica Sep-Pak cartridges (600 mg silica) were purchased from Waters Associates (Milford, MA). Boron trifluoride etherate and dimethyl sulfoxide (DMSO) were purchased from Aldrich Chemical (Milwaukee, WI). All other reagents were the highest grade available.

Methods

FGM preparation

Two major approaches were used to isolate FGM. Approach A was based on churning the cream and precipitating the proteins of the serum with ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$. Several variations of this procedure were tried (Preparations A-1, A-2, A-3). Approach B involved the combined extraction and delipidation of FGM by DMSO with the simultaneous removal of milk fat by centrifugation (Preparation B). Milk from Guernsey cows was used for all preparations, except A-3, because of the high cream content of milk produced by this breed. Preparation A-3 was made from milk obtained from a mixed herd that contained Holstein, Ayrshire and Brown Swiss breeds.

Preparation A-1. This procedure was based on the method of Herald & Brunner (1957). Cream was separated from raw milk (within 2 h of milking) at 40°C with a small DeLaval separator, washed four times with three volumes of deionized water at the same temperature and stored overnight at 4°C. The next day, the chilled cream was churned with a Brinkmann homogenizer (Model PT20ST) until separation of butterfat and serum was observed. The mixture was heated to 45°C and centrifuged at $52\,000 \times g$. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the serum to a final concentration of 2.2 M; the solution was stirred for 1 h at room temperature and centrifuged at $27\,500 \times g$ for 1 h at 5°C. To remove neutral lipids of the fat globule that might remain associated with the membrane, the pellets were homogenized by hand in 35% ethanol:65% diethyl ether, stirred for 30 min, and filtered through Whatman #114 paper. Operations were carried out in jacketed glassware with ice water cooling. The membrane was washed five times with diethyl ether at 0°C and then three times with diethyl ether at 30°C, for 10 min, 5 min and 3 min, respectively, decanting and discarding the solvent each time. Residual ether was removed by evaporation under nitrogen using a rotary evaporator; FGM was stored at -20°C.

Preparation A-2. After the cream was separated as in Preparation A-1 and washed once with water, aliquots of cream were stirred with three volumes of cold 0.01 M tris-0.25 M sucrose buffer, pH 7.5, and centrifuged at $13\,700 \times g$ for 35 min at 2°C. Pooled solid cream was diluted with an equal volume of 4°C water and churned with the Brinkmann homogenizer. Then the resulting mixture was warmed to 45°C and centrifuged at $115\,000 \times g$ for 1 h at 4°C. The recovered membrane pellets were then treated as in Preparation A-1. After removal

of residual ether by rotary evaporation, the FGM was stored at -20°C .

Preparation A-3. The following variation of the method of Swope & Brunner (1970) was used. Fresh raw milk was warmed to 45°C and separated with the small DeLaval separator. The cream was washed with three to four volumes of distilled water at 45°C and re-separated; this process was repeated until the wash water was clear. The washed cream was chilled at 8°C overnight, adjusted to about 40% milkfat, and churned in a Waring blender. The broken emulsion (buttermilk and butterfat) was warmed to 45°C and separated with the DeLaval separator. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the buttermilk to a final concentration of 2.2 M and the mixture was centrifuged at $20\,000 \times g$ at ambient temperature for 30 min. The combined pellets were stored at 8°C . For lipid analysis, the pellets were dialyzed against deionized water at 5°C until sulfate was no longer present in the dialysate when tested with BaCl_2 ; FGM was stored at -20°C .

Preparation B. FGM was prepared using an adaptation of the method of Dapper *et al.* (1987). Cream was separated from raw milk (within 2 h of milking) as in Preparation A-1, washed once with three volumes of 40°C deionized water, separated again, and cooled to 5°C . Aliquots of cream were stirred with three volumes of 0.01 M tris-0.25 M sucrose, pH 7.5, and centrifuged at $20\,000 \times g$ for 30 min at 2°C . Solidified washed cream was pooled and stored at 5°C . For FGM isolation, 5–12 g samples of solidified cream were weighed into polyallomer centrifuge tubes. A 50% solution (v/v) of DMSO in deionized water was added to each tube at a ratio of about 6.6 ml/g of cream; tubes were centrifuged at $70\,000 \times g$ for 1.5 h at 2°C . Solid butter oil was removed from the top, the clear DMSO solution decanted, and the upper portion of the centrifuge tube cut off to provide access to the compact FGM pellet. Combined pellets were stored at -20°C .

Lipid analysis

Lipid extraction. Total lipids of 1–2 g samples of FGM were extracted with chloroform:methanol (2:1 v/v) at a ratio of 25 ml per gram of FGM. After removal of the precipitated proteins by filtration, solvent was removed from the lipid extract on a rotary vacuum evaporator, and the extract was dried in a vacuum desiccator to constant weight. The lipid extract was then redissolved in chloroform:methanol (2:1 v/v) to obtain a solution containing 25 mg of lipids/ml of solvent. Neutral and polar

lipids were separated (Fig. 1) on prepacked silica Sep-Pak cartridges using a modification of literature methods (Juaneda & Rocquelin, 1985; Hamilton & Comai, 1988). The bulk FGM lipid solution (1 ml) was applied onto a silica cartridge, previously washed with 20 ml of chloroform, via an attached 5-ml syringe. The neutral lipids and free fatty acids were then eluted by washing the cartridge with 20 ml of chloroform. Polar lipids were eluted subsequently with 20 ml of chloroform:methanol (2:1 v/v). Solvent was removed from each fraction under vacuum, and the fractions were then placed in a vacuum desiccator until constant weight was attained. The neutral and polar fractions were reconstituted to a concentration of 5 mg/ml in chloroform and chloroform:methanol (2:1 v/v), respectively.

High-performance liquid chromatography. The HPLC system consisted of a Hewlett Packard (Avondale, PA) Model 1090 solvent delivery module equipped with a Rheodyne (Rainin, Woburn, MA) Model 7125 loop injector with a 20 μ l loop and a Tracor (Austin, TX) Model 945 flame ionization detector (FID). The stainless steel column, 4.6 mm \times 10 cm, was packed with Rainin Microsorb silica, 3-micron particle size. The

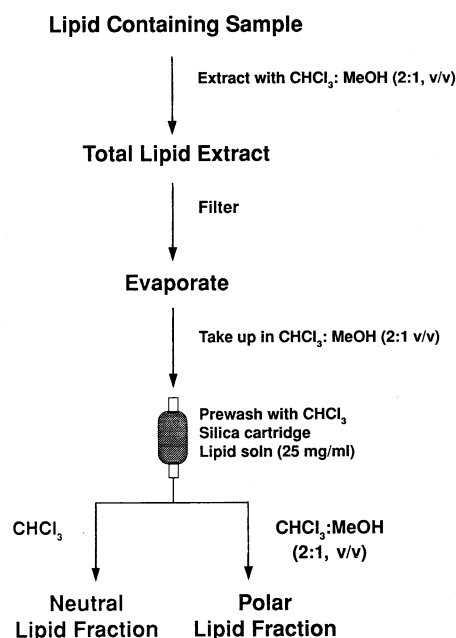


Fig. 1. Separation of neutral and polar lipid fractions from total fat globule membrane lipids on silica cartridge. Procedure described in the text.

FID output was routed to a Hewlett-Packard Model 3393A integrator to determine retention times and peak areas.

Class separation of neutral lipids into cholesterol esters, free fatty acids, triglycerides, diglycerides, monoglycerides, and cholesterol was obtained using the following gradient elution profile at a flow rate of 0.8 ml/min: initial solvent composition isooctane:isopropanol (99.5:0.5 v/v); hold for 2 min; then program to isooctane:isopropanol (97:3 v/v) over 3 min; hold for 10 min; return to original conditions over 3 min. Class separation of the polar lipid fractions was carried out following a modification of earlier procedures (Grieser & Geske, 1989; Becart *et al.*, 1990; Moreau *et al.*, 1990). Briefly, the polar lipid fraction was separated on the HPLC column with a mobile phase consisting of chloroform, methanol, water, and ammonium hydroxide. Gradient elution was as follows: solvent A was CHCl₃:MeOH:28% NH₄OH (80:19.5:0.5 v/v/v) and solvent B was CHCl₃:MeOH:H₂O:28% NH₄OH (60:34.5:5.0:0.5 v/v/v/v). Initial conditions were 100% A; program to 100% B over 14 min; hold for 4 min; return to 100% A over 5 min. The column must be re-equilibrated for 10 min prior to the next injection to obtain the proper separations. Quantification of FGM lipids was obtained from calibration curves established for each class of neutral and polar lipids as described previously (Maxwell *et al.*, 1987).

Gas chromatography. The instrument was a Hewlett Packard (Avondale, PA) Model 5895 gas chromatograph equipped with a split capillary injector and a flame ionization detector. Separations were obtained using a fused silica capillary column, 60 m × 0.25 mm I.D., coated with SP-2340 (Supelco, Bellefonte, PA). Determinations were made using He as carrier gas (1 ml/min) with a split ratio of 80:1 and the following oven temperature program: initial temperature 140°C; then 0.5°C/min to 155°C; then 2°C/min to 200°C; hold for 20 min. Methyl heneicosonate (C_{21:0}) served as internal standard. Signal analysis was accomplished by routing the detector output to a Hewlett-Packard Model 3396A integrator. Fatty acyl methyl esters (FAMEs) of glycerides (about 10 mg) from the total lipid extract and both neutral and polar lipid fractions were prepared as previously described (Marmer & Maxwell, 1981) by transesterification with boron trifluoride/methanol.

RESULTS AND DISCUSSION

The total lipid contents of FGM preparations analyzed in this study are given in Table 1. As shown, three of the four methods of FGM preparation examined (A-1, A-2, and B) produced the same level of total

TABLE 1
Lipid Composition of Bovine Fat Globule Membrane

<i>FGM preparation</i>	<i>Total lipids, (%)^a</i>	<i>Neutral lipids, (%)^b</i>	<i>Polar lipids, (%)^b</i>
A-1	6.4	20.8	79.2
A-2	5.9	17.7	82.3
A-3	13.8	72.7	27.3
B	6.0	18.1	81.9

^aWeight % of FGM; total lipid extracted with 2:1 CHCl₃:MeOH.

^bIsolated by class separation using a silica cartridge.

lipid (approximately 6 weight % of FGM), whereas FGM A-3 produced twice the amount of lipid (approximately 14 weight %).

Subsequent separation of FGM total lipids into their neutral and polar lipid classes was accomplished on silica cartridges, as shown in Fig. 1. This technique, previously used for the separation of serum lipids (Juaneda & Rocquelin, 1985), was modified by washing the silica cartridges with chloroform prior to use. Failure to prewash the silica cartridges, regardless of source, gave variable results because of contaminants that often co-elute with the neutral lipid fraction. After prewashing, approximately 25 mg of lipid were applied onto the silica cartridge in 1 ml of chloroform:methanol (2:1 v/v). Previous work has shown that void volume of the columns is 1.5 ml and that column capacity for lipids is approximately 50 mg (Juaneda & Rocquelin, 1985; Hamilton & Comai, 1988). Using this concentration of lipid (25 mg in 1 ml of solvent), therefore, avoids column overload or breakthrough of lipid from the cartridge.

As shown in Table 1, the ratio of polar to neutral lipids for FGM Preparations A-1, A-2, and B was about 4:1, whereas for FGM Preparation A-3 this ratio was reversed to about 1:4. The latter ratio indicated that a significant carryover of neutral lipids occurred in this method of FGM preparation. This was confirmed upon HPLC analysis of the neutral and polar lipid fractions. The separation of these fractions into their respective subclasses was accomplished on the silica HPLC column. The separations obtained are shown in Fig. 2. Neutral lipid class separations (Panel A, Fig. 2) were obtained using a mobile phase consisting of isooctane:isopropanol with the gradient elution profile described in the Experimental section. The conditions of this separation were specifically developed for this study and have not been described previously.

In carrying out the analysis it is important that the initial mobile phase

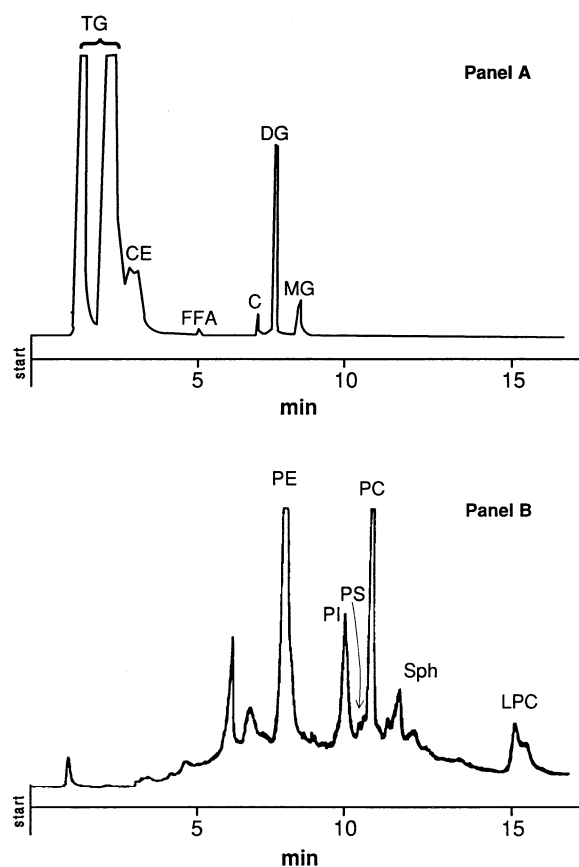


Fig. 2. HPLC separation of fat globule membrane lipids. Panel A: neutral lipid species, designations as in Table 2. Panel B: polar lipid species, designations as in Table 3.

composition be 99.5:0.5 (v/v) since above or below this composition triglycerides and cholesterol esters co-elute. It should also be noted in Fig. 2, Panel A, that the triglycerides associated with FGM, as well as milk fat, elute as two distinct peaks in the chromatogram. This is a result of the high content of short chain acyl residues in milk fat triglycerides (Keenan *et al.*, 1970); moreover, tricaproin (a C_{18} triglyceride) eluted under the conditions given above in the later triglyceride peak while tripalmitin (a C_{48} triglyceride) eluted in the earlier triglyceride peak.

Polar lipid class separations (Panel B, Fig. 2) were obtained using a slight modification of the procedure described by Becart *et al.* (1990). Under these experimental conditions, separation and quantification of phosphatidylserine from phosphatidylcholine was the most difficult since the former eluted just prior to the latter. Nevertheless, compositional

TABLE 2
Neutral Lipids of Bovine Fat Globule Membrane

Neutral lipids	% of total ^a	Literature values	
		Sharma ^b	Thompson ^c
Triglycerides	70.3 ^d	67.4	67.1
Diglycerides	14.3	12.5	10.2
Monoglycerides	2.8	5.6	5.9
Free fatty acids	2.6	6.9	7.9
Cholesterol	3.7	4.9	6.5
Cholesterol esters	1.8	2.7	1.0
Others ^e	4.5	—	1.4

^aAverage area % neutral lipids of FGM as determined by HPLC. SD = $\pm 0.7\%$.

^bAverage weight % as calculated from enzymatic values (Sharma *et al.*, 1987).

^cAverage weight % FGM as calculated from silicic acid chromatography values (Thompson *et al.*, 1961).

^dPreparation A-3 contained significantly higher percentage of neutral lipids as triglycerides and is, therefore, excluded.

^eIncludes carotenoids, squalene, or unidentified components.

analysis of the polar lipids can be obtained if the HPLC procedure is followed in detail.

Table 2 shows the percentage composition of each neutral lipid subclass of FGM and compares the results with data obtained by enzymatic (Sharma *et al.*, 1987), and silicic acid chromatographic (Thompson *et al.*, 1961) methods. Data typical for three methods of FGM preparation (A-1, A-2, and B) are similar to the literature data (Table 2). Preparation A-3, on the other hand, gave higher and more variable values than those shown in Table 2. Accordingly, it was concluded that triglyceride carryover accounted for the high total lipid content observed for FGM Preparation A-3 in Table 1.

Analytical results for the polar lipid fraction isolated from FGM preparations are given in Table 3; data obtained by enzymatic (Sharma *et al.*, 1987) and thin layer chromatographic (TLC) (Keenan *et al.*, 1970) methods are shown for comparison. Data for phospholipids of skim milk obtained by HPLC (Christie *et al.*, 1987) are also included to indicate the similarity when using HPLC methodology. The HPLC data for polar lipid subclasses reported here compare favorably with those obtained by TLC (Keenan *et al.*, 1970) and enzymatic (Sharma *et al.*, 1987) methods, with the following minor difference. The polar lipids

isolated from FGM contained comparable amounts of phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine but lesser amounts of phosphatidylinositol. However, data here compare favorably with the HPLC data of Christie *et al.* (1987). The sphingomyelin content was similar for all methods listed in Table 3.

A detailed fatty acid compositional analysis of all lipid groups for three methods of FGM Preparations (A-1, A-2, and B) is listed in Table 4. The methodology of this study provided, for the first time, extensive data for the fatty acid compositions of the neutral and polar fractions separately, as well as for total FGM lipids. Preparation A-3 was omitted because its neutral lipid content is not representative of the other methods of FGM preparations. The percentage fatty acyl composition data of total FGM lipids is typical of that of milk fat (Keenan *et al.*, 1970), as characterized by the relatively large amounts of C_{14:0}, C_{16:0}, C_{18:0}, and C_{18:1 ω 9} fatty acids and the relatively high saturated to unsaturated fatty acyl ratio of about 2.4:1. A more informative analysis of FGM lipids was obtained by examining the fatty acyl composition of the neutral and polar lipid fractions separately. These data show that the phospholipid acyl groups contain relatively lesser amounts of C_{14:0} and C_{16:0} and a larger amount of C_{18:1 ω 9} compared with the neutral lipid fraction. Moreover, the phospholipid fraction, as expected, contained a higher

TABLE 3
Polar Lipids Distribution of Bovine Fat Globule Membrane

Phosphatides	% ^a	Literature values (%)		
		Sharma ^b	Keenan ^c	Christie ^d
Phosphatidylethanolamine	35.7	33.3	26.8	34.2
Phosphatidylinositol	5.7	6.1	10.4	6.2
Phosphatidylserine	4.9	13.7	11.2	2.8
Phosphatidylcholine	26.8	27.5	28.0	25.4
Sphingomyelin	21.4	19.4	21.4	21.4
Lysophosphatidylcholine	2.2	—	2.2	—
Other ^e	3.3	—	—	10.0

^aAverage area % polar lipids of FGM determined by HPLC (SD = $\pm 0.5\%$).

^bAverage weight % as calculated from enzymatic values (Sharma *et al.*, 1987).

^cAverage weight % as calculated from thin layer chromatography values (Keenan, *et al.*, 1970).

^dAverage weight % polar lipids in skim milk as determined by HPLC (Christie *et al.*, 1987).

^eContained ceramide sugars and unidentified compounds.

TABLE 4
Fatty Acyl Composition of Bovine Fat Globule Membrane^a

Fatty acyl residue	Retention time (min) ^b	Lipid (wt. %) ^c		
		Total	Neutral	Polar
C _{8:0}	5.58	0.89	0.67	0.20
C _{10:0}	6.36	0.40	0.35	0.34
C _{12:0}	7.82	3.02	2.71	0.72
C _{14:0}	10.42	10.12	11.64	2.48
C _{14:1}	12.05	0.27	0.35	0.40
C _{15:0}	12.42	0.49	0.93	0.60
C _{16:0}	15.11	36.27	36.55	15.04
C _{16:1ω7}	17.11	0.57	0.68	0.79
C _{17:0}	18.41	0.90	0.75	0.50
C _{18:0}	22.66	15.52	24.28	23.10
C _{18:1ω9}	24.29	24.76	19.11	40.85
C _{18:1ω7}	25.37	0.80	0.25	0.72
C _{19:0}	27.32	0.12	0.06	0.20
C _{18:2ω6}	29.01	1.61	0.68	9.64
C _{20:0}	32.40	0.46	0.31	0.46
C _{18:2ω3}	33.99	0.26	0.18	0.52
C _{20:4ω4}	43.50	0.12	0.09	0.53
Unidentified	—	3.42	1.41	2.90
Sat./Unsat.	—	2.40	3.85	0.82

^aFor FGM preparations A-1, A-2, and B.

^bOrder of elution from GC column SP2340. Details in Experimental section.

^cArea weight % relative to internal standard (heneicosanoate).

proportion of unsaturated fatty acyl residues as reflected by a saturated to unsaturated ratio of approximately 1:1, compared with the neutral lipid fraction ratio of approximately 4:1.

The foregoing results indicate that FGM prepared with procedures A-1, A-2, and B contained significantly less neutral lipid than FGM from the more traditional procedure A-3. This has prime importance when further study of FGM proteins is desired because excess neutral lipid interferes with protein isolation and purification. Moreover, the ease with which Preparation B is accomplished suggests that it could be the method of choice. This preparative method has been used successfully in investigating the role of glycopospholipid anchors in FGM proteins (Malin & Basch, 1990) and in isolating and purifying alkaline phosphatase from FGM preparations (unpublished data).

REFERENCES

- Basch, J. J., Farrell, H. M., Jr & Greenberg, R. (1976). Identification of the milk fat globule proteins. I. Isolation and partial characterization of glycoprotein B. *Biochim. Biophys. Acta*, **448**, 589-98.
- Basch, J. J., Greenberg, R. & Farrell, H. M., Jr (1985). Identification of the milk fat globule proteins. II. Isolation of major proteins from electrophoretic gels and comparison of their amino acid compositions. *Biochim. Biophys. Acta*, **830**, 127-35.
- Becart, J., Chevalier, C. & Biesse, J. P. (1990). Quantitative analysis of phospholipids by HPLC with a light scattering evaporating detector — application to raw materials for cosmetic use. *J. High Resol. Chromatog.*, **13**, 126-29.
- Christie, W. W., Noble, R. C. & Davies, G. (1987). Phospholipids in milk and dairy products. *J. Soc. Dairy Technol.*, **40**, 10-12.
- Dapper, C. H., Valivullah, H. M. & Keenan, T. W. (1987). Use of aprotic solvents to release membranes from milk lipid globules. *J. Dairy Sci.*, **70**, 760-65.
- Freudenstein, C., Keenan, T. W., Eigel, W. N., Sasaki, M., Stadler, J. & Franke, W. W. (1979). Preparation and characterization of the inner coat material associated with fat globule membranes from bovine and human milk. *Exp. Cell Res.*, **118**, 277-94.
- Grieser, M. D. & Geske, J. N. (1989). High performance liquid chromatography of phospholipids with flame ionization detection. *J. Am. Oil Chem. Soc.*, **66**, 1484-7.
- Hamilton, J. G. & Comai, K. (1988). Rapid separation of neutral lipids, free fatty acids and polar lipids using prepacked silica Sep-Pak columns. *Lipids*, **23**, 1146-9.
- Heid, H. W., Winter, S., Bruder, G., Keenan, T. W. & Jarasch, E.-D. (1983). Butyrophilin, an apical plasma membrane-associated glycoprotein characteristic of lactating mammary glands of diverse species. *Biochim. Biophys. Acta*, **728**, 228-38.
- Herald, C. T. & Brunner, J. R. (1957). The fat-globule membrane of normal cow's milk. I. The isolation and characteristics of two membrane-protein fractions. *J. Dairy Sci.*, **40**, 948-56.
- Juaneda, P. & Rocquelin, G. (1985). Rapid and convenient separation of phospholipids and non-phosphorus lipids from rat heart using silica cartridges. *Lipids*, **20**, 40-1.
- Kanno, C. & Kim, D.-H. (1990). A simple procedure for the preparation of bovine milk fat globule membrane and a comparison of its composition, enzymatic activities, and electrophoretic properties with those prepared by other methods. *Agric. Biol. Chem.*, **54**, 2845-54.
- Keenan, T. W. & Huang, C. M. (1972). Membranes of mammary gland. VI. Lipid

- and protein endoplasmic reticulum from bovine mammary gland. *J. Dairy Sci.*, **55**, 1586-96.
- Keenan, T. W., Morré, D. J., Olson, D. E., Yunghans, W. N. & Patton, S. (1970). Biochemical and morphological comparison of plasma membrane and milk fat globule membrane from bovine mammary gland. *J. Cell Biol.*, **44**, 80-93.
- Malin, E. L. & Basch, J. J. (1990). Differential release of proteins from bovine fat globule membrane. *Biochem. Cell Biol.* **68**, 899-902.
- Mangino, M. E. & Brunner, J. R. (1977). Isolation and partial characterization of xanthine oxidase associated with the milk fat globule membrane of cow's milk. *J. Dairy Sci.*, **60**, 841-50.
- Marmer, W. N. & Maxwell, R. J. (1981). Dry column method for the quantitative extraction and simultaneous class separation of lipids from muscle tissue. *Lipids*, **16**, 365-71.
- Maxwell, R. J., Nungesser, E. H., Marmer, W. N. & Foglia, T. A. (1987). HPLC with flame ionization detection: class separation, linearity of response, and quantification of sterols, glycerides, and phospholipids. *LC-GC*, **5**, 829-33.
- Moreau, R. A., Asmann, P. T. & Norman, H. A. (1990). Analysis of major classes of plant lipids by high-performance liquid chromatography with flame ionization detection. *Phytochem.*, **29**, 2461-6.
- Morton, R. K. (1953). Alkaline phosphatase of milk. 2. Purification of the enzyme. *Biochem. J.*, **55**, 795-800.
- Patton, S. & Keenan, T. W. (1975). The milk fat globule membrane. *Biochim. Biophys. Acta*, **415**, 273-309.
- Sharma, K. C., Kumari, A., Sareen, V. K. & Singh, S. (1987). Lipid composition of globule membranes from butter milk and butter serum of buffalo and cow milk. *Milchwissenschaft*, **42**, 439-42.
- Swope, F. C. & Brunner, J. R. (1970). Characteristics of the fat globule membrane of cow's milk. *J. Dairy Sci.*, **53**, 691-9.
- Thompson, M. P., Brunner, J. R., Stine, C. M. & Lindquist, K. (1961). Lipid components of the fat-globule membrane. *J. Dairy Sci.*, **44**, 1589-96.